Nuclear Magnetic Resonance Detection in Capillary Electrophoresis

See **II / ELECTROPHORESIS / Capillary Electrophoresis-Nuclear Magnetic Resonance**

One-dimensional Polyacrylamide Gel Electrophoresis

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Electrophoresis is based on the differential migration of electrically charged particles in an electric field. As such, the method is applicable only to ionic or ionogenic materials, i.e. substances convertible to ionic species (a classic example being neutral sugars, which form negatively charged complexes with borate). In fact, with the advent of capillary zone electrophoresis (CZE) it has been found that a host of neutral substances can be induced to migrate in an electric field by inclusion in charged micelles, e.g. of anionic (sodium dodecyl sulfate, SDS) or cationic (cetyltrimethylammonium bromide, CTAB) surfactants. Even compounds that are not ionic, ionogenic, or complexable can often be analysed by CZE as they are transported past the detector by the strong electroosmotic flow on the capillary walls.

Basically, if one plots the velocity of a zone against the pH in the same zone, electrophoretic techniques can be divided into four main types: zone electrophoresis (ZE) together with moving-boundary electrophoresis (MBE), discontinuous (disc) electrophoresis, isotachophoresis (ITP) and isoelectric focusing (IEF). **Figure 1** represents this classification. It can be seen that IEF and ITP are based on principles that are 'perpendicular' to ZE and MBE. In particular, in IEF, once steady-state conditions have been attained, all proteins reach a zero-velocity (v) thus they remain immobile ($v = 0$, pH-axis). It is then clear that ITP closes the ring of possibilities: all zones move with the same velocity, but at different pH. Alternatively, electrophoretic techniques may be enumerated in chronological order, as follows: moving boundary electrophoresis (MBE), zone electrophoresis (ZE), disc electrophoresis, isoelectric focusing (IEF), sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE), two-dimensional (2-D) maps, isotachophoresis (ITP), staining techniques, immobilized pH gradients (IPG), and capillary zone electrophoresis.

ZE became a reality when hydrophilic gels (acting as an anticonvective support) were discovered. Grabar and Williams in 1953 first proposed the use of an agar matrix (currently abandoned in favour of a highly purified agar fraction, agarose). They also combined, for the first time, electrophoresis on a hydrophilic support with biospecific detection (immunoelectrophoresis). Barely two years after that, Smithies (1955) applied another gel, potato starch. The starch blocks were highly concentrated matrices (12–14% solids) and subsequently introduced a new parameter in electrophoretic separations: molecular sieving. Human sera, which in cellulose acetate or paper electrophoresis, were resolved in barely five bands, now produced a spectrum of 15 zones. The most important discovery, however, came with the introduction of polyacrylamide gels and disc electrophoresis; this discovery was thoroughly debated in

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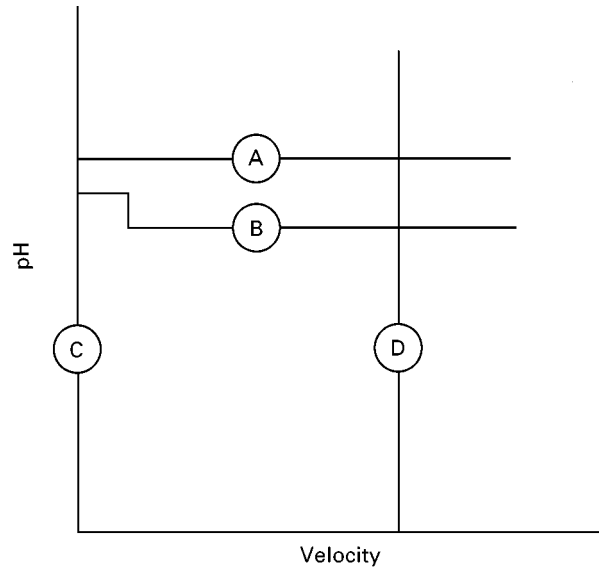

Figure 1 Classification of the four modes of electrokinetic techniques. The velocity of a zone is plotted against the pH in the same zone. (A) zone and moving boundary electrophoresis; (B) discontinuous electrophoresis; (C) isoelectric focusing; (D) isotachophoresis. (Reproduced with permission from Routs RJ (1971) PhD thesis, University of Eindhoven.

a classic electrophoretic volume, which appeared in December, 1964 in the *Annals of the New York Academy of Sciences* (a collectors item!). This was like the explosion of a supernova in the firmament of electrokinetic methodologies. Although most of the above-mentioned techniques belong to the category of one-dimensional PAGE, we will only mention three of them here in detail: disc electrophoresis, SDS electrophoresis and pore-gradient-gel electrophoresis. The other techniques such as IEF and ITP, being steady-state methods, are best performed in non-sieving media. The technique of moving-boundary electrophoresis died out long ago.

Discontinuous Electrophoresis

In 1959 Raymond and Weintraub described the use of polyacrylamide gels (PAG) in ZE, which offered UV and visible transparency (starch gels are opalescent) and the ability to sieve macromolecules over a wide range of sizes. **Figure 2** gives a scheme of reaction for producing polyacrylamide gels from the standard mixture of monomers, acrylamide and the cross-linker Bis. It should be noted that although this matrix should be neutral (except where accidental hydrolysis of acrylamide to acrylic acid occurs), in reality it is not completely devoid of charges; at the

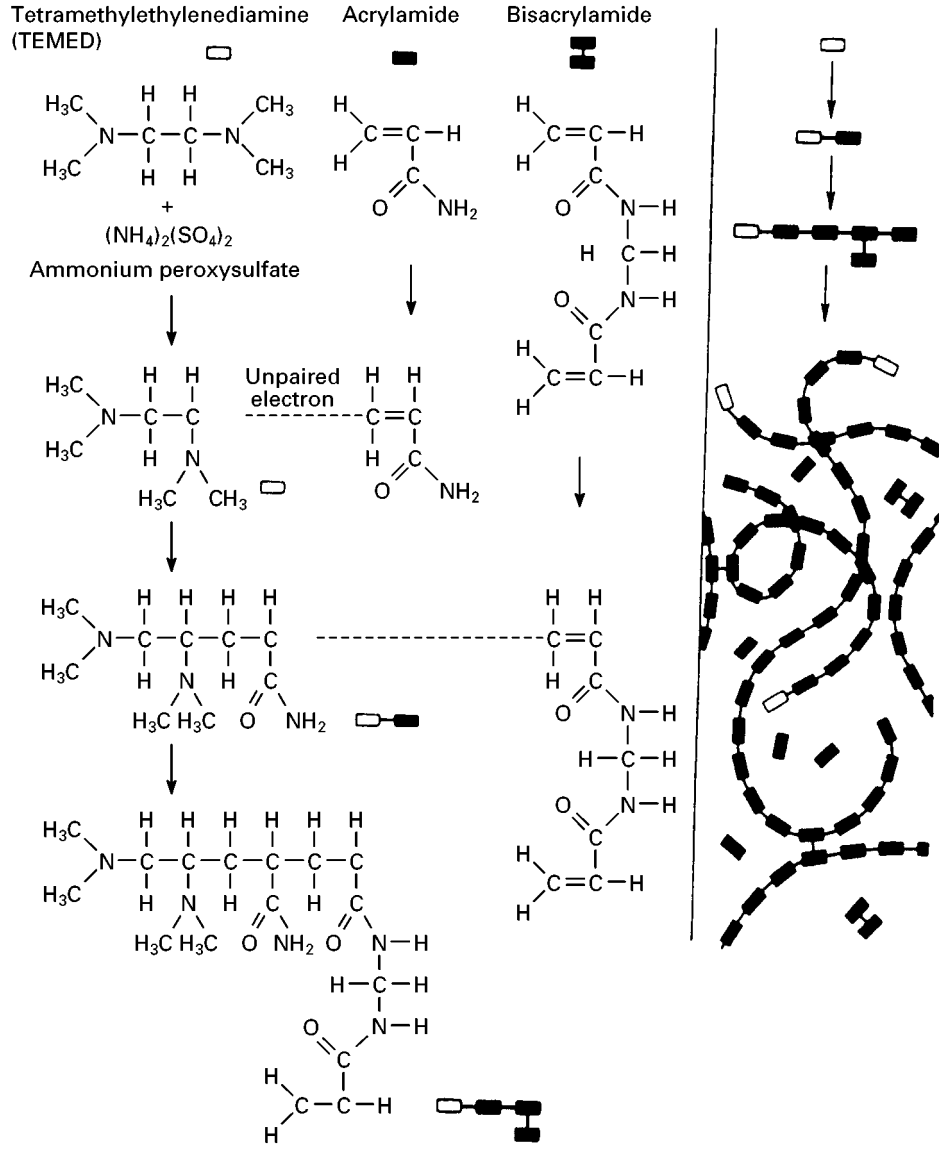

Figure 2 The polymerization reaction of acrylamide. The chemical formula of acrylamide, *N,N′*-methylenebisacrylamide (Bis) and of the initiators (peroxysulfate and *N,N,N′,N′*-tetramethylethylendiamine, TEMED) are shown. On the right-hand side, growing polyacrylamide chains, in equilibrium with free monomers, are illustrated. In this particular case, it is assumed that the chain termini are TEMED molecules, although peroxysulfate could be just as well incorporated.

chain termini either initiator, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), or sulfate, could be incorporated which would impart positive or negative charges, respectively. The fact that polyacrylamides always exhibit a residual electroosmotic flow towards the cathode suggest that an excess of negative charges is incorporated over positive ones (TEMED).

In 1964, Ornstein and Davis created discontinuous (disc) electrophoresis by applying to PAG a series of discontinuities (of leading and terminating ions, pH, conductivity, and porosity), thus further increasing the resolving power of the technique. In discontinuous disc electrophoresis (the principle of which is outlined in **Figure 3**), the proteins are separated on the basis of two parameters: surface charge and molecular mass. The matrix is divided into three sections (from bottom up): a 'separation', or 'running' gel, a 'spacer' or 'stacking' gel, and a sample gel. A sharp discontinuity exists at the running/stacking interface: the bottom gel is a tightly knit sieve (with small pores), while the second and third layers are minimally sieving, open-pore structures. At the same interface, a second discontinuity exists in pH. In fact, the running gel is titrated at pH 8.9, whereas spacer and sample gels are buffered at pH 6.7. This gel region at pH 6.7 is also a low conductivity region (third discontinuity), which means that a voltage gradient will be generated in this zone when an electric current is passed through it. Below and above it (in the cathodic chamber) high-conductivity regions are found. A fourth discontinuity exists at the interface between the upper gel end and the liquid in the cathodic compartment: below it only Cl^- (leading, L) ions are present, while above it only glycinate (trailing or terminating, T) ions are found.

Why is there the need for such a complicated system? This intricate set up must satisfy the Kohlrausch regulating function, which is at the heart of ITP (in fact, movement of ions in the first two gel segments will be according to ITP rules). If all the ions in the system are arranged in such a way that $\mu_L > \mu_P > \mu_T$ (where μ is the mobility of leading, protein and terminating ions, respectively), then, upon playing a voltage gradient, they will migrate down the gel cylinder with equal velocities and the boundary between each adjacent species will be maintained. As soon as the electric circuit is closed, Cl^- (fastest moving) ions are swept down the column towards the anode. Just behind this boundary, all protein ions will start arranging themselves in order of their mobilities, with the lowest p*I* component next to the Cl^- boundary and

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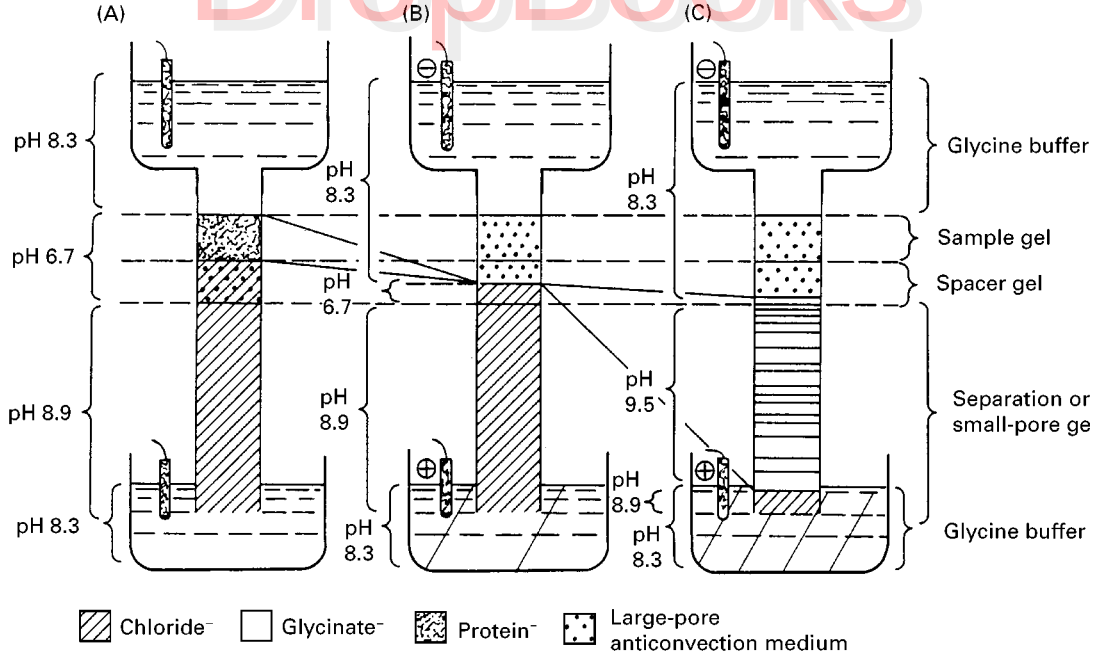

Figure 3 Principle of discontinuous disc electrophoresis. (A) Sample in sample gel; (B) sample concentration in stacking gel; (C) sample separation in running gel. From top to bottom, the following phases are encountered: glycine buffer at pH 8.3 in the cathodic reservoir; sample gel and spacer gel, both titrated to pH 6.7; small-pore running gel, titrated to pH 8.9; and glycinate buffer again in the anodic reservoir at the bottom. In part (C) it is seen that, as the glycinate boundary sweeps down the gel past the protein zones, the pH increases from 8.9 (in A and B) up to 9.5. (Reproduced with permission from Ornstein, 1964.)

the highest p*I* species closing the procession. The last 'wagon of the train' is the glycinate (terminating) ion; and explains why the sample and spacer gels are titrated at pH 6.7. Gly has a theoretical p*I* of 6.1 but, as shown by its titration curve, it is almost isoelectric, even at pH 6.7; its anionic mobility, therefore, is extremely small, in any event smaller than the slowest protein ion.

Thus, in the sample and spacer gels, two basic phenomena occur: (1) all protein ions are sorted out and physically separated according to their p*I*s, and (2) each protein ion is strongly concentrated in extremely thin starting zones (the disc barely a few micrometres thick and a concentration process of up to 1000- to 10 000-fold). This isotachophoretic 'train', however, does not have a long life; as it enters the running gel, the train 'runs off the tracks'. Only the 'locomotive' (Cl^-) of the train is unaffected; the various protein wagons now overrun each other, since they experience a strong frictional force, due to the highly sieving matrix, so that now their velocity is a function of their charge/size ratio. In addition, as the almost isoelectric Gly enters the pH 8.9 zone, its negative charge density strongly increases so that it jumps ahead and closely follows the Cl^- ion. As Gly sweeps down the running gel, the pH increases from pH 8.9 to 9.5 (approaching the p*K* value of the Gly amino group) so that now the net charge on Gly is -0.5. As a consequence of this further jump in pH, all proteins experience an additional mobility increment. One might wonder why, after taking on such an experimental burden in forming the ITP train, one should then destroy it and continue the run in the plain zone electrophoretic mode. There are reasons for this. First, the ITP train, while maintaining high resolution due to lack of degradation of zone boundaries, has the main defect that the zones are contiguous and continuous, i.e. they are not separated by blank zones of plain buffer. As a result, when staining the gel, one would only see a single, continuous zone of protein ions, with no visible separation between zones. Second, whereas the sharp protein discs formed during the stacking (ITP) process are separated solely by surface charge, during migration in the running gel, separations continue on the basis of an additional parameter i.e. the mass. The small loss of resolution due to diffusion of the protein discs in the running gel is more than compensated for by the resolution increments due to size (coupled to charge) fractionation in this gel zone. Although disc electrophoresis is no longer in vogue, it was an extremely useful analysis technique for at least 20 years after its inception. Moreover, the general principle has not been abandoned and it is used today as a stacking technique in both SDS and capillary electrophoresis.

Disc electrophoresis could also be used for deriving physico-chemical parameters of the proteins under analysis. In 1964, Ferguson showed that one can derive parameters which are proportional to both the surface charge and the mass of the macromolecule. This can be accomplished by plotting the results of a series of experiments with polyacrylamide gels of varying porosity. For each protein under analysis, the slope of the curve log m_T (electrophoretic mobility) vs gel density (%T) is proportional to molecular mass, while the y-intercept (Y_0) is a measure of surface charge. Examples of these plots are shown in **Figure 4**. In **Figure 4**A the two parallel lines indicate charge isomers; in Figure 4B, the fanning out lines indicate a family of constant charge and different mass; in **Figure 4**C, the two crossing lines indicate proteins differing in both charge and mass. Recently, non-linear Ferguson plots have been reported (Chrambach, 1988), related to the reptation mode of DNA in sieving media.

Sodium Dodecyl Sulfate (SDS) Electrophoresis

SDS electrophoresis fractionates polypeptide chains essentially on the basis of their size. It is therefore a simple, yet powerful and reliable, method for molecular mass determination. In 1967 Shapiro *et al.* first reported that electrophoretic migration in SDS is proportional to the effective molecular radius and, thus, to the M_r of the polypeptide chain. This means that SDS must bind to proteins and cancel out differences in molecular charge, so that all components will migrate solely according to size. Surprisingly, large amounts of SDS appear to be bound (an average of 1.4 g SDS/g protein). This means that the number of SDS molecules bound is of the order of half the number of amino acid residues in a polypeptide chain. This amount of highly charged surfactant molecules is sufficient to overwhelm effectively the intrinsic charges of the polymer coil, so that their net charge per unit mass becomes approximately constant. If migration in SDS (and disulfide-reducing agents, such as 2-mercaptoethanol, in the denaturing step, for a proper unfolding of the proteins) is proportional only to M_r, then, in addition to cancelling out charge differences, SDS also equalizes molecular shape differences (e.g. globular vs rod-shaped molecules). This seems to be the case for protein–SDS mixed micelles. These complexes can be assumed to behave as ellipsoids of constant minor axis (*c.* 1.8 nm) and a major axis proportional to the length of the amino acid chain (i.e. to molecular mass) of the protein. The rod length for the 1.4 g SDS/g protein

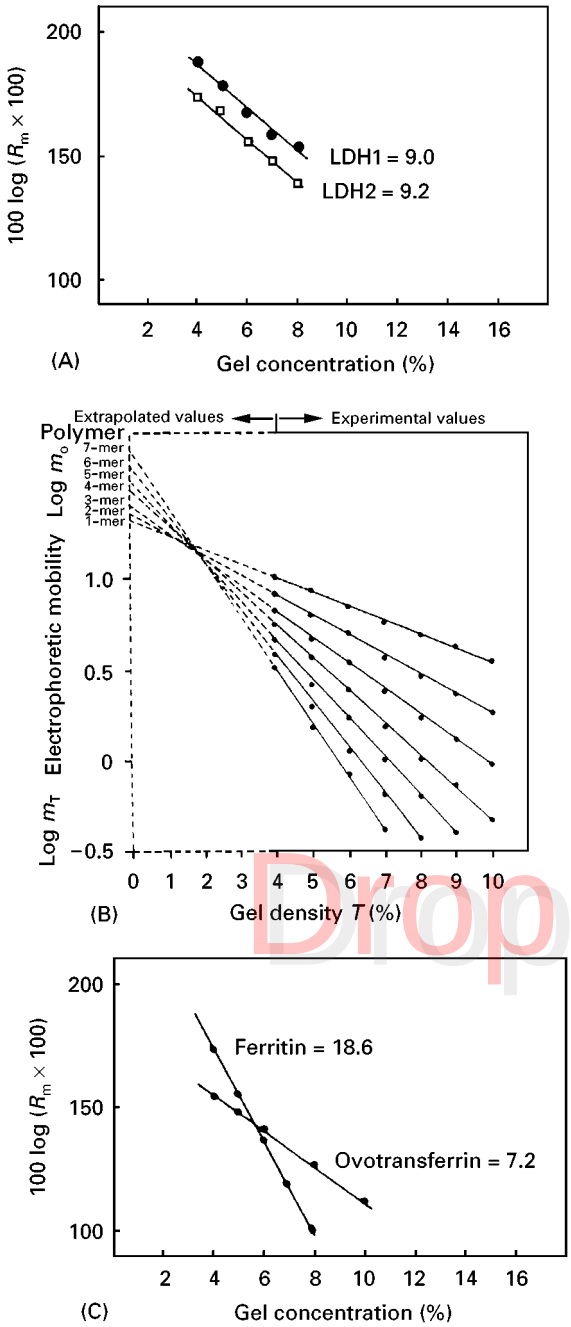

Figure 4 Ferguson plots (log R_m, relative mobility, vs %T, total monomer concentration) in the case of: (A) lactic dehydrogenase (LDH) 1 and 2 (isomers of charge, exhibiting the same mass); (B) serum albumin (polymeric forms, from monomer to heptamer, having constant charge and pure size difference, since all curves meet in gel-free environment, at 2% T where polyacrylamide will liquefy); (C) ferritin and ovalbumin, two totally unrelated proteins differing in both size and charge. (Parts (A) and (C) reproduced with permission from Hedrick and Smith, 1968 and Part (B) from Thorun, 1971.)

complex is of the order of 0.074 nm per amino acid residue. For further information on detergent properties, see Helenius and Simons (1975).

In SDS electrophoresis, the proteins can be prelabelled with dyes that covalently bind to their $-NH_2$ residues. The dyes can be conventional, like the blue dye Remazol, or fluorescent, such as dansyl chloride, fluorescamine, *O*-phthaldialdehyde, and MDPF (2-methoxy-2,4-diphenyl-3[2H]-furanone). Prelabelling is compatible with SDS electrophoresis, as the size increase is minimal, but would be anathema in disc electrophoresis or IEF, as it would generate a series of bands of slightly altered mobility or p*I* from an otherwise homogeneous protein. Although at its inception SDS electrophoresis used continuous buffers, today the preferred set up is via discontinuous buffers and matrices, simplified from the original disc electrophoresis assembly (see **Figure 5**). This ensures much higher resolving power, due to formation of ultrathin protein zones.

For treatment of data, the sample and M_r standards are electrophoresed side-by-side in a gel slab. After detection of the polypeptide zones, the migration distance (or R_F) is plotted against log M_r to produce a calibration curve (Neff *et al.*, 1981) from which the M_r of the sample can be calculated (see **Figure 6**). It should be noted that in a gel of constant %T, linearity is obtained only in a certain range of molecular sizes. Outside this limit a new gel matrix of appropriate porosity should be used. Two classes of proteins show anomalous behaviour in SDS electrophoresis: glycoproteins (because their hydrophilic oligosaccharide units prevent hydrophobic binding of SDS micelles) and strongly basic proteins, e.g. histones (because of electrostatic binding of SDS micelles through their sulfate groups). The first anomaly can be partially alleviated by using alkaline Tris/borate buffers, which will increase the net negative charge on the glycoprotein and thus produce migration rates well correlated with molecular size. The migration of histones can be improved by using pore-gradient gels and allowing the polypeptide chains to approach the pore limit.

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Porosity Gradient Gels

When macromolecules are electrophoresed in a continuously varying matrix concentration (which results in a porosity gradient) rather than in a gel of uniform concentration, the protein zones are compacted along their track, as the band front is, at any given time, at a gel concentration somewhat higher than that of the rear of the band, so that the former is decelerated continuously. A progressive band sharpening thus results. There are other reasons for resorting to gels of graded porosity. We have seen that disc electrophoresis separates macromolecules on the basis of both size and charge differences. If the influence of molecu-

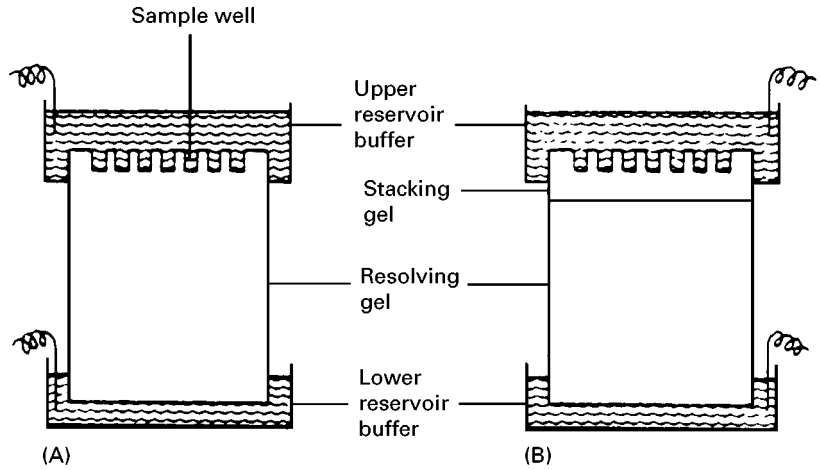

Figure 5 Typical set up of a gel slab for SDS electrophoresis in a continuous (A) or discontinuous (B) gel and buffer system. In both cases, the sample is applied as a dense liquid layer in pockets precast in the gel slab by the teeth of the comb. Note that this mode of sample deposition avoids the use of the third gel phase, the sample gel, as typically adopted in disc electrophoresis.

lar charge could be eliminated, then clearly the method could be used with a suitable calibration for measuring molecular size. This has been accomplished by overcoming charge effects in two main ways. In one such way, a relatively large amount of charged ligand, such as SDS, is bound to the protein, effectively swamping the initial charges present on the protein molecules and giving a quasi-constant charge-to-mass ratio. However, in SDS electrophoresis, proteins are generally dissociated into their constituent polypeptide subunits, and the concomitant loss of functional integrity and antigenic properties cannot be prevented. Therefore, the size of the original, native molecule must be evaluated in the absence of denaturing substances.

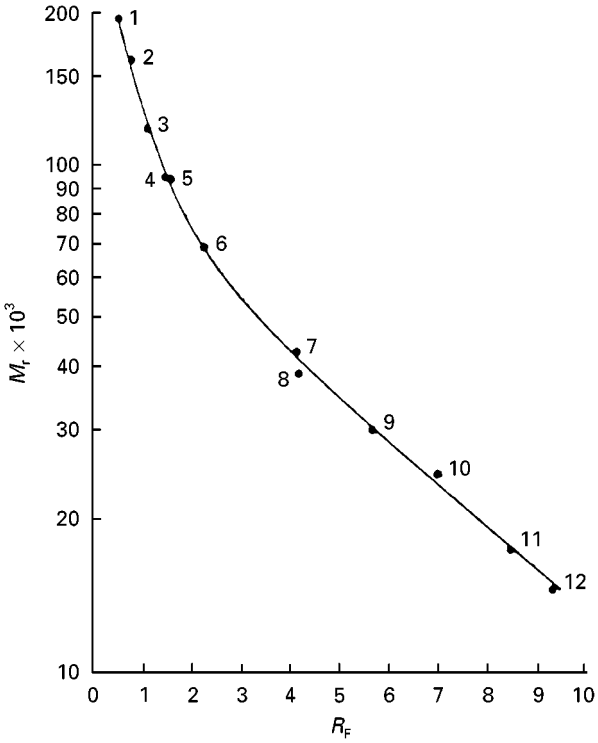

Figure 6 Typical log M_r vs R_F plot after an SDS-PAGE run. Note that the plot is linear only in the M_r 15 000–60 000 Da range. The M_r markers are: (1) myosin (194 000 Da); (2) RNA polymerase (β-β-subunit, 160 000 Da); (3) β-galactosidase (116 000 Da); (4) phosphorylase B (94 000 Da); (5) RNA polymerase (δ-subunit; 95 000 Da); (6) bovine serum albumin (68 000 Da); (7) ovalbumin 43 000 Da); (8) RNA polymerase (α-subunit; 38 400 Da); (9) carbonic anhydrase (30 000 Da); (10) trypsinogen (24 500 Da); (11) β-lactoglobulin (17 500 Da); and (12) lysozyme (14 500 Da). (Reproduced with permission from Neff *et al.*, 1981.)

In the second method for M_r measurement, this can be done by relying on a mathematical cancelling of charge effects, following measurement of the mobility of native proteins in gels of different concentrations. This is the so-called 'Ferguson plot' discussed above. As a third method for molecular size measurements one can use gels of graded porosity. This method is characterized by high resolving power and relative insensitivity to variability in experimental conditions. See **Figure 7** for a typical experimental set up for casting porosity gradients in gel slabs. Under appropriate conditions (at least 10 kV × hours), the mobility of most proteins becomes constant and eventually ceases as each constituent reaches a gel density region in which the average pore size approaches the diameter of the protein (pore limit) (Margolis and Kenrick, 1968). Thus, the ratio between the migration distance of a protein to that of any other becomes a constant after the proteins have all entered a gel region in which they are subjected to drastic sieving conditions. This causes the electrophoretic pattern to become constant after prolonged migration in a gel gradient. The gel concentration at which the migra-

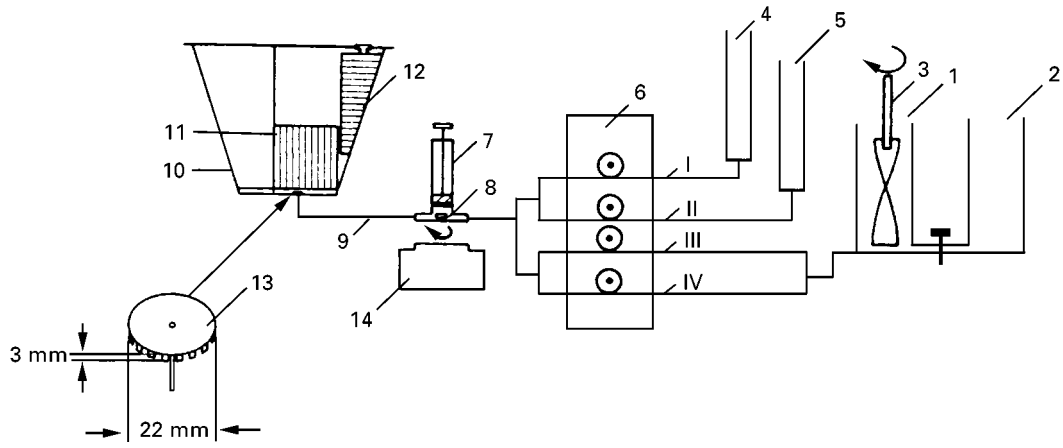

Figure 7 Scheme of the apparatus used for the simultaneous preparation of eight gradient gel slabs. (1) and (2) two-chamber mixer; (3) stirrer; (4) reservoir for peroxosulfate; (5) reservoir for TEMED; (6) proportioning pump; (7) modified disposable syringe, used as small chamber mixer; (8) magnetic bar; (9) tube connecting the stirrer to the gel casting apparatus (10); (11) gel cassettes; (12) wedge; (13) distributor; (14) magnetic stirrer; I and II, 0.5 mm i.d. vinyl tubings; III and IV, 3.16 mm i.d. vinyl tubings. (Reproduced with permission from Rothe and Purkhanbaba, 1982.)

tion rate for a given protein becomes constant is called the 'pore limit'. If this porosity is properly mapped with the aid of a suitable set of marker proteins, it is possible to correlate the migration distance to the molecular mass of any constituent in the mixture.

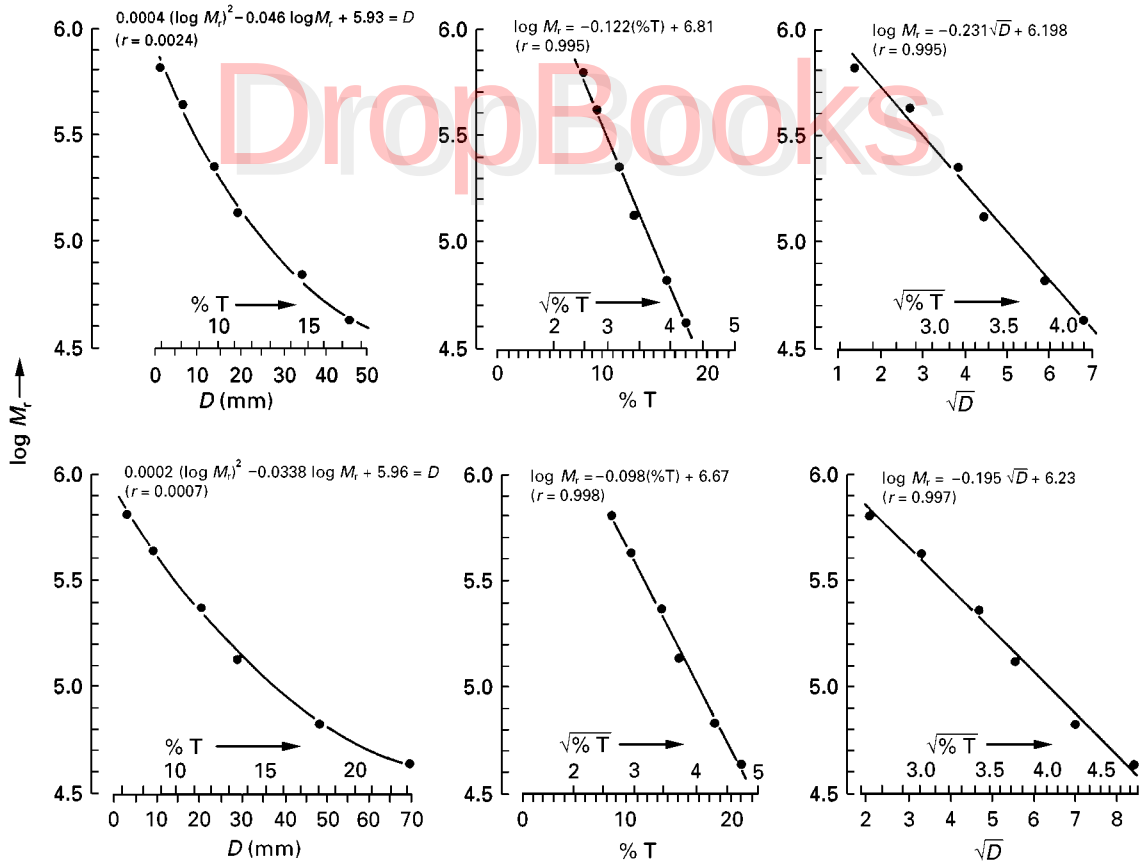

Figure 8 Typical log M_r vs migration distance (D) or gel composition (%T) plots after pore-gradient electrophoresis. Note that these plots are non-linear, whereas when log M_r is plotted against $\sqrt{D}$ or $\sqrt{\%T}$ a linear relationship is obtained. (Reproduced with permission from Rothe and Purkhanbaba, 1982.)

After electrophoresis has finished, the experimental data gathered can be handled in two ways: a two-step or a one-step method. The most promising two-step approach appears to be that of Lambin and Fine (1979), who observed that there is a linear relationship between the migration distance of proteins and the square root of electrophoresis time, provided that time is kept between 1 and 8 h. The slopes of the regression lines of each protein in the above graph are an indication of molecular size. When the slopes of the various regression lines thus obtained are plotted against the respective molecular masses, a good linear fit is obtained, which allows M_r measurements of proteins of between 2×10^5 and 10^6 Da. The shape of the proteins (globular or fibrillar), their carbohydrate content (up to 45%) and their free electrophoretic mobilities (between 2.1 and 5.9×10^{-5} $cm^2\ V^{-1}\ s^{-1}$) do not seem to be critical for proper M_r measurements by this procedure. One-step methods have been described by Rothe and Purkhanbaba (1982). These authors found that when log M_r is plotted against either D (distance migrated) or %T (acrylamide + Bis), a nonlinear correlation is always obtained. However, when log M_r is plotted vs $\sqrt{\%T}$ or $\sqrt{D}$, a linear regression line is obtained, which allows the accurate determination of M_r values of proteins (standard deviation of $\pm 3.7\%$; see **Figure 8**. The correlations log $M_r - \sqrt{\%T}$ or log $M_r - \sqrt{D}$ are not significantly altered by the duration of electrophoresis. Therefore, a constant M_r value should be obtained for a stable protein, no matter how long electrophoresis takes. More recently, Rothe and Maurer (1986) have demonstrated that the relationship log M_r vs $\sqrt{D}$ is also applicable to SDS electrophoresis in linear polyacrylamide gel gradients.

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